

Original Article

Silencing Notch-1 enhances fludarabine chemosensitivity in chronic lymphocytic leukemia

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Abstract: Objective: Notch signaling pathways are essential for cell proliferation, differentiation, and apoptosis. Fludarabine is widely used in the treatment of chronic lymphocytic leukemia (CLL). The present study aimed to investigate the effects of Notch-1 siRNA and fludarabine on cell growth and chemosensitivity in CLL cells. Materials and methods: Human CLL cell line MEC-1 was treated with Notch-1 siRNA and fludarabine. In the Notch-1 siRNA plus fludarabine group, Notch1 siRNA was transfected into MEC-1 cells before fludarabine treatment. Expression of Notch-1 mRNA and protein levels were determined by Quantitative Real-Time Polymerase Chain Reaction and Western blotting, respectively. Cell proliferation and apoptosis were detected by MTT assay and flow cytometry, respectively. Moreover, this study measured expression of anti-apoptotic factor Bcl-2 and pro-apoptotic protein Bax. Results: Treatment with 1.6 µg/mL of fludarabine for 24 hours and Notch-1 siRNA-6150 were used. Notch-1 siRNA effectively downregulated expression of Notch-1 in CLL cells. Both downregulation of Notch-1 by siRNA and fludarabine treatment suppressed cell growth and increased cell apoptosis. The combination of Notch-1 siRNA and fludarabine led to an enhancement in growth inhibition and apoptosis rates in CLL cells, relative to single treatment. Notch-1 siRNA and fludarabine treatment induced a significant increase in expression of Bax and a decrease in expression of Bcl-2 in CLL cells. Notch siRNA synergistic fludarabine further increased the expression change of Bax and Bcl-2. Discussion: Cell growth inhibition caused by Notch-1 siRNA and fludarabine treatment might be related to cell apoptosis induction. Downregulation of Notch-1 may increase chemosensitivity to fludarabine in CLL.

Keywords: Chronic lymphocytic leukemia, Notch-1, fludarabine, small interference RNA

Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common hematological malignancies in adults, worldwide [1]. It is characterized by proliferation of aberrant monoclonal B lymphocytes with defective cell death mechanisms in peripheral blood and bone marrow, lymph nodes, and the spleen [2-4]. Incidence of CLL has increased yearly, likely due to the advancement of diagnostic methods and environmental aspects. CLL is a heterogeneous disease with a significant variation in disease progression [5]. Although current advancements have improved remission rates and long-term survival of patients, CLL remains an incurable malignant blood disease [4, 6, 7]. Development of effective therapeutic targets will largely contribute to the treatment of CLL.

Fludarabine, a purine analogue and cell cycle non-specific drug, has been widely used in the treatment of CLL, inhibiting DNA repair in resting cells and DNA synthesis in dividing cells [8]. Fludarabine has been considered the most effective drug in treating CLL and is recommended as the frontline therapy [9]. Notch signaling pathway plays an essential role in regulating various cell processes. Aberrant regulation of Notch signaling pathways will lead to abnormal tissue development, along with occurrence of tumors and other diseases [10, 11]. Accumulating evidence has indicated that inhibition of Notch-1 expression in tumor cells can increase cell apoptosis and inhibit cell proliferation [12, 13]. Nefedova et al. [14] indicated that inhibition of Notch signaling prevented drug resistance and enhanced sensitivity to chemotherapy of cancer cells. However, cross-talk

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between fludarabine and Notch signaling pathways has not been demonstrated.

In the present study, researchers investigated the effects of silencing Notch-1 and fludarabine treatment on cell growth and apoptosis in CLL. Moreover, this study explored whether down regulation of Notch signaling could sensitize CLL cells to fludarabine chemotherapeutics.

Materials and methods

Cell culture and treatment

Human CLL cell line MEC-1 was cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO₂ humidified atmosphere. Cells were plated and cultured in 6-well plates. After reaching 80% confluence, cells were treated with scrambled siRNA, Notch-1 siRNA, fludarabine, and Notch-1 siRNA + fludarabine, respectively. To determine the effects of concentrations and exposure times of fludarabine, cells were incubated with various concentrations (0.2 µg/mL, 0.4 µg/mL, 0.8 µg/mL, 1.6 µg/mL, 3.2 µg/mL) of fludarabine for 24 hours, 48 hours, and 72 hours, respectively. After treatment, cells were harvested for further experimentation.

Small interference RNA (siRNA) treatment

Three Notch-1 siRNA sequences (Notch-1 siRNA-2010, siRNA-6150, and siRNA-780) were used to examine the efficiency of silencing Notch-1 mRNA expression in MEC-1 cells. Specific sequences for three Notch-1 siRNAs were as follows: Notch-1 siRNA-2010 forward: 5'-CAGGGAGCAUGUGUAACAUTT-3' and reverse: 5'-AUGUUACACAUGCUCUGTT-3'; Notch-1 siRNA-6150 forward: 5'-GGGCUAACAAGAUUGCAATT-3' and reverse: 5'-UGCAUAUCUUUGUAGCCCTT-3'; Notch-1 siRNA-780 forward: 5'-GUCAGGAAACAACUGCAATT-3' and reverse: 5'-UUGCAGUUGUUCCUGGACTT-3'. Negative control siRNA (forward: 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse: 5'-ACGUGACACGUUCGGAGAATT-3') did not match any known mammalian gene bank sequences. MEC-1 cells were transfected with Notch-1 siRNAs or control siRNA that had been precomplexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Measurement of cell viability

Cell viability was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [15]. Briefly, MEC-1 cells were plated and cultured in 96-well plates. Cells in scrambled siRNA and Notch-1 siRNA groups were transfected with scrambled siRNA and Notch-1 siRNA, respectively. Cells in the fludarabine group were treated with fludarabine. Cells in the Notch-1 siRNA + fludarabine group were transfected with Notch-1 siRNA before fludarabine treatment. After washing twice with PBS, the cells were incubated in the presence of MTT for 4 hours at 37°C. MTT solution was then removed and replaced with 10% dimethylsulfoxide (DMSO) to solubilize formazan product at 37°C for 6 minutes. Optical density of the active cells was quantified at 540 nm.

Flow cytometric detection for cell apoptosis

Cells (with a density of 5×10⁵) were harvested by trypsinization and fixed using 4% paraformaldehyde at 4°C, overnight, to detect cell apoptosis. Cells were washed twice with cold PBS and re-suspended in 50 µl pre-cooling binding buffer and 5 µl 7-amino-actinomycin D (7-AAD) dye solution. This was followed by incubation at room temperature for 15 minutes under dark conditions. After the addition of 450 µl binding buffer, 1 µl Annexin V-phycoerythrin (PE) dye solution was added. Cells were then incubated under dark conditions at room temperature for 15 minutes. Apoptotic cells were annexin V-PE positive and 7-AAD negative. Samples were placed on a flow cytometer (Beckman Coulter, Chicago, USA) to measure apoptotic rates.

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

TRIzol (Beyotime, Jiangsu, China) was used to extract total RNA of the cells. cDNA was generated using Prime Script RT-PCR Kit (Thermo, MAS, USA) on Roche 480 system (Roche, Dubai, UAE), following manufacturer instructions. Synthesized first-strand cDNA was used as template and β-actin was applied as a normalization for PCR amplification, respectively. Standard curves and PCR results were analyzed using Light Cycler 480 real-time PCR instrumentation. The following primers were used:

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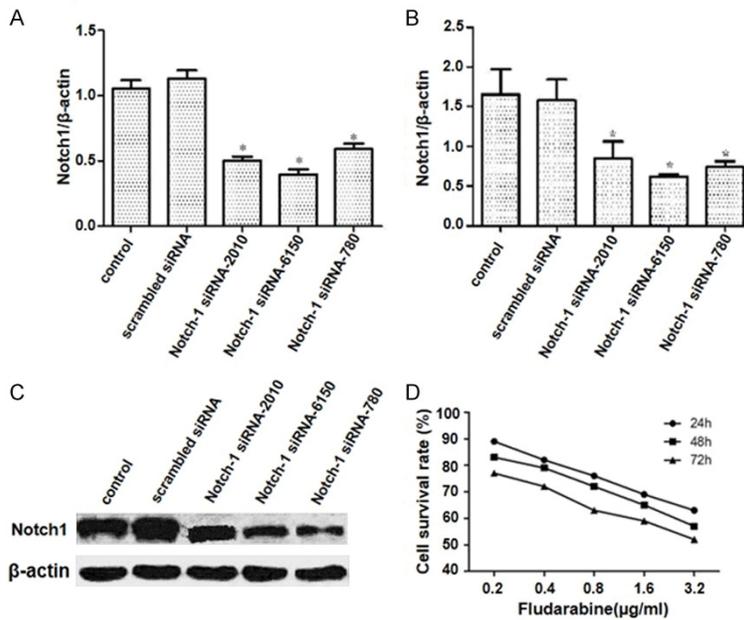


Figure 1. Notch-1 siRNA downregulates expression of Notch-1 in CLL cells and fludarabine treatment suppresses CLL cell proliferation. A. Notch-1 siRNA effectively downregulates expression of Notch-1 at the mRNA level in CLL cells. B. Notch-1 siRNA suppresses expression of Notch-1 at the protein level in CLL cells. C. Notch-1 protein levels were measured by Western blot. All three Notch-1 siRNAs significantly downregulated expression of Notch-1 in CLL cells and Notch-1 siRNA-6150 showed the highest interfering efficiency. * $P < 0.05$. D. Fludarabine suppresses cell proliferation in a dosage-dependent manner in CLL cells. Treatment with 1.6 $\mu\text{g/ml}$ of fludarabine for 24 hours resulted in moderate cell injury for MEC-1 cells (with a cell viability of 67.2%).

Notch-1 forward: 5'-GGCACTTTCTGTGAGGAGG-A-3' and reverse: 5'-GCAGTCAGGCGTGTGTTCTT-3'; Bcl-2 forward: 5'-GAGGATTGTGGCCTTCTT-3' and reverse: 5'-CCAGGAGAAATCAAACAGAG-3'; Bax forward: 5'-CTGAGCAGATCATGAAGACA-3' and reverse: 5'-CTCTGCAGCTCCATGTTACT-3'; β -actin forward: 5'-CACTGTGCCATCTACGA-3' and reverse: 5'-TGATGTCACGCACGATTT-3'.

Western blotting

Western blotting was implemented for quantification of protein expression. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Cwbiotech, Beijing, China) containing a protease inhibitor. Total protein concentrations were measured by BCA assay (Cwbiotech, Beijing, China). Protein samples were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then were transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk at

room temperature for 1 hours, followed by incubation with primary antibodies against Notch-1 (1:1000, Santa Cruz, CAL, USA), Bax (1:1000, Santa Cruz, CAL, USA), Bcl2 (1:1000, Santa Cruz, CAL, USA), and β -actin (1:1000, Santa Cruz, CAL, USA) at 4°C overnight. Afterward, the membrane was washed with Tris-buffered saline with 0.1% Tween-20 (TBST) three times and incubated with anti-rabbit or anti-mouse IgG conjugated horseradish peroxidase secondary antibody (1:5000, Zhongshan Jinqiao Biotechnology, Beijing, China) at room temperature for 1 hour. Protein bands were detected using an enhanced chemiluminescence detection kit (Millipore, Billerica, USA) and were analyzed by laser densitometry with Multi Gauge version 4.0 (Fujifilm Life Science, Tokyo, Japan). Quantitative analysis was normalized to β -actin. All experiments were performed at least three times.

Statistical analysis

SPSS 20.0 software (SPSS Inc., Armonk, NY, USA) was used to conduct statistical analysis. Data are presented as mean \pm standard deviation. All statistical tests were analyzed by two-way ANOVA. Statistical significance is defined as $P < 0.05$. Graphs were drawn using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

Notch-1 siRNA effectively downregulates expression of Notch-1 in CLL cells

To evaluate the effects of siRNA on Notch signaling pathways, three types of Notch-1 siRNAs (Notch-1 siRNA-2010, siRNA-6150, and siRNA-780) were used. This study initially evaluated the interfering efficiency of different siRNAs on expression of Notch-1 at mRNA and protein levels. After transfection with three Notch-1 siRNAs and control siRNA for 48 hours, cells were collected to examine the expression of Notch-1

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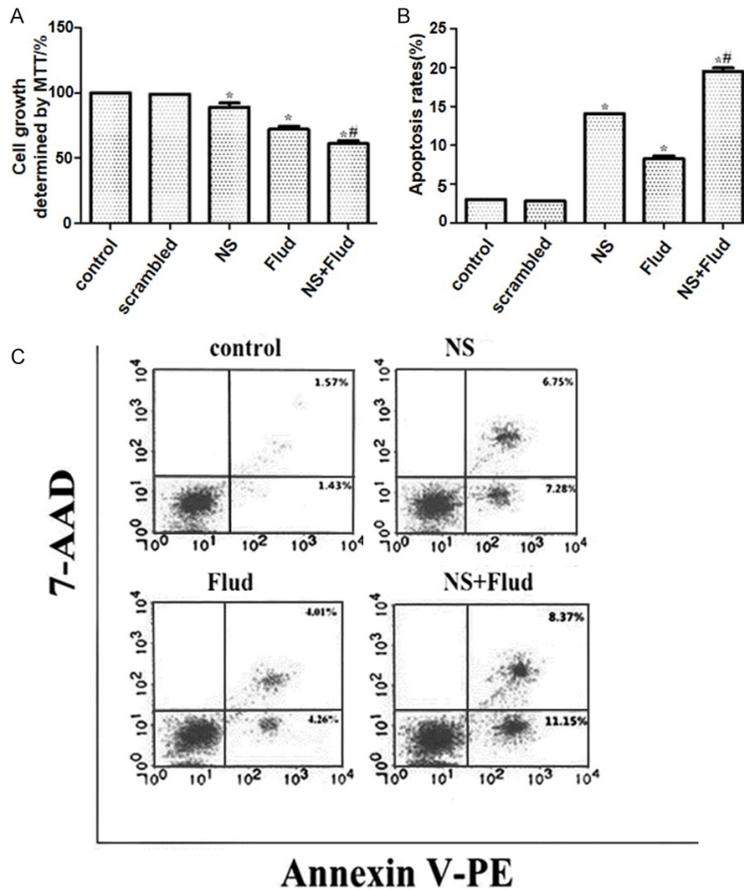


Figure 2. Downregulation of Notch-1 by siRNA increases fludarabine chemosensitivity. A. Silencing Notch-1 and fludarabine treatment effectively inhibited cell proliferation by MTT array. The combination of Notch-1 siRNA and fludarabine led to an enhancement in growth inhibition relative to single treatment in CLL cells. B, C. Notch-1 siRNA and fludarabine treatment significantly increased cell apoptosis. The combination of Notch-1 siRNA with fludarabine increased cell apoptosis rates in CLL cells. * $P < 0.05$ relative to control group, $\#P < 0.05$ relative to single treatment. NS: Notch-1 siRNA, Flud: fludarabine.

at mRNA and protein levels using qRT-PCR and Western blot analysis, respectively. Results showed that all three Notch-1 siRNAs significantly downregulated expression of Notch-1 at both mRNA (Figure 1A) and protein levels (Figure 1B and 1C) in CLL cells. Of these, Notch-1 siRNA-6150, showing the highest interfering efficiency, was chosen for subsequent analysis.

Fludarabine suppresses cell proliferation in a dosage-dependent manner in CLL cells

MEC-1 cells were incubated with 0.2 $\mu\text{g/mL}$, 0.4 $\mu\text{g/mL}$, 0.8 $\mu\text{g/mL}$, 1.6 $\mu\text{g/mL}$, and 3.2 $\mu\text{g/mL}$ of fludarabine for 24 hours, 48 hours, and 72 hours, respectively. Results revealed that fludarabine suppressed cell proliferation in a

dosedependent manner (Figure 1D). Treatment of MEC-1 cells with 1.6 $\mu\text{g/mL}$ of fludarabine for 24 hours resulted in moderate cell injury (with a cell viability of 67.2%). Thus, treatment with 1.6 $\mu\text{g/mL}$ of fludarabine for 24 hours was used in subsequent studies.

Downregulation of Notch-1 by siRNA increases fludarabine chemosensitivity

Effects of Notch-1 siRNA and fludarabine on cell growth were evaluated by MTT assay. Data showed that both downregulation of Notch-1 and fludarabine treatment resulted in a reduction of MEC-1 cell growth ($P < 0.05$, Figure 2A). Combination of Notch-1 siRNA and fludarabine treatment led to an obvious enhancement in growth inhibition relative to single treatment in MEC-1 cells ($P < 0.05$, Figure 2A). The influence of Notch-1 siRNA and fludarabine on cell apoptosis was evaluated by flow cytometric detection. Flow cytometry analysis demonstrated that cell apoptosis was significantly increased after Notch-1 siRNA or fludarabine treatment ($P < 0.05$, Figure 2B and 2C). Growth inhibition induced by Notch-1 siRNA and fludarabine might be partially attributed to the increase of

cell apoptosis. Moreover, to further verify the mechanisms of combined effects, this study investigated cell apoptosis rates induced by fludarabine after Notch-1 siRNA transfection. Flow cytometry showed that the combination of Notch-1 siRNA with fludarabine increased cell apoptosis rates in CLL cells relative to single treatment ($P < 0.05$, Figure 2B and 2C). Present data suggests that Notch-1 siRNA transfected MEC-1 cells were more sensitive to fludarabine-induced apoptosis.

Notch-1 siRNA and fludarabine treatment up-regulates Bax and downregulates Bcl-2 in CLL cells

To further clarify the mechanisms of enhanced cell apoptosis of CLL cells induced by Notch-1

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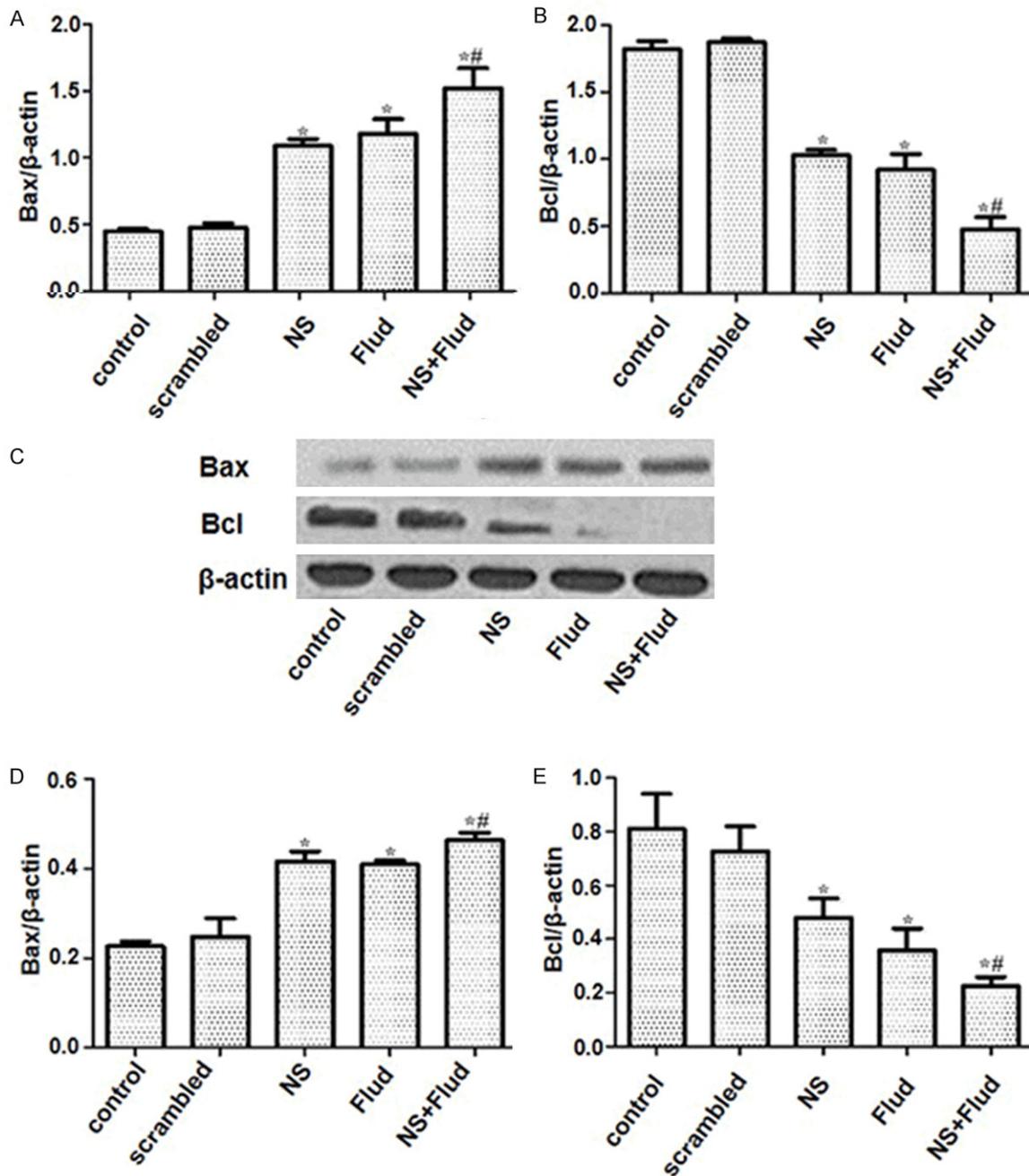


Figure 3. Notch-1 siRNA and fludarabine treatment upregulates pro-apoptotic protein Bax and downregulates anti-apoptotic factor Bcl-2 in CLL cells. A, B. Notch-1 siRNA and fludarabine treatment induce a significant increase of Bax mRNA levels and a significant decrease of Bcl-2 mRNA levels. Notch siRNA synergistic fludarabine further increase the changes of Bax and Bcl-2 mRNA levels. C-E. Notch-1 siRNA and fludarabine treatment induce a significant increase of Bax protein levels and a significant decrease of Bcl-2 protein levels. Notch siRNA synergistic fludarabine further increase the changes of Bax and Bcl-2 protein levels. * $P < 0.05$ relative to control group, # $P < 0.05$ relative to single treatment. NS: Notch-1 siRNA, Flud: fludarabine.

siRNA and fludarabine, this study investigated expression of anti-apoptotic factor Bcl-2 and pro-apoptotic protein Bax. It was found that Notch-1 siRNA and fludarabine treatment induced

a significant increase of Bax mRNA levels and a significant decrease of Bcl-2 mRNA levels in MEC-1 cells. Notch siRNA synergistic fludarabine further increased changes of Bax and Bcl-2

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mRNA levels (**Figure 3A** and **3B**). Protein level changes of Bax and Bcl-2 were consistent with mRNA levels (**Figure 3C-E**). Results indicate that fludarabine drug sensitivity enhanced by silencing Notch-1 was related to downregulation of anti-apoptotic factor Bcl-2 and upregulation of pro-apoptotic protein Bax.

Discussion

Results of the present study showed that both downregulation of Notch-1 by siRNA and fludarabine treatment suppressed cell growth and increased cell apoptosis. Downregulation of Notch-1 was correlated with chemosensitivity of fludarabine in CLL. Additionally, silencing Notch-1 and fludarabine treatment downregulated anti-apoptotic factor Bcl-2 and upregulated pro-apoptotic protein Bax.

Notch signaling pathways play an important role in cell proliferation, differentiation, survival, and apoptosis. Notch signal disorders, interacted with other signal pathways, can lead to the occurrence of tumors in a variety of ways. Previous studies have shown that abnormal activation of Notch signaling pathways is associated with the development of various cancers, such as breast cancer [16], glioma [17], nasopharyngeal carcinoma [18], and colon cancer [19]. A member of Notch family, Notch-1 is most commonly detected in tumor tissues [20]. Abnormal expression of Notch-1 plays an important role in the regulation of tumor growth, metabolism, and cell apoptosis. Regulation of Notch-1 may be considered a novel target for treatment of CLL. To explore the effects of Notch-1 during the development of CLL, expression of Notch-1 in MEC-1 cells was interfered using siRNAs. Results showed that silencing Notch-1 in MEC-1 cells suppressed cellular proliferation and promoted cell apoptosis, consistent with previous studies [11, 21, 22].

Fludarabine has been widely used in the treatment of CLL. Previous studies have indicated that fludarabine treatment could increase complete remission rates and overall response rates of CLL patients [23, 24]. Fludarabine plus cyclophosphamide is the recommended first-line treatment of CLL. However, drug resistance is still an important issue in the treatment of patients with CLL. Notch signaling pathways play a critical role in the development and progression of tumors. This might be a new therapeutic target for the treatment of CLL. Previous studies have indicated that silencing Notch-1 enhanced the sensitivity of drugs on tumors

[12, 14]. The present study investigated whether silencing Notch-1 could affect the viability of CLL cells and the sensitivity to fludarabine chemotherapy. In this experiment, the synergistic effects of silencing Notch-1 and fludarabine treatment on CLL cells were studied. Present results confirmed the role of Notch-1 in the pathogenesis of CLL, finding that silencing Notch-1 enhanced CLL cell sensitivity to fludarabine.

Previous studies have shown that Bax and Bcl-2 are involved in the apoptosis of tumor cells and downregulation of Bax or upregulation of Bcl-2 can reverse the apoptosis effects of drugs [25-27]. The present study demonstrated that cell apoptosis was increased, along with the upregulation of Bax and downregulation of Bcl-2. It was also found that both silencing Notch-1 and fludarabine treatment increased Bax expression and reduced Bcl-2 expression at mRNA and protein levels. Therefore, results suggest that silencing Notch-1 can enhance fludarabine-induced cell apoptosis, possibly mediated by Notch pathways via regulation of Bcl-2 family expression.

Conclusion

The present study demonstrated that cell growth inhibition caused by Notch-1 siRNA and fludarabine treatment might be associated with cell apoptosis induction. Moreover, downregulation of Notch-1 could enhance chemosensitivity to fludarabine in CLL. Future studies should combine the inhibitors of Notch molecules with neoplastic drugs, aiming to further investigate the roles of Notch signaling in chemotherapeutic drug sensitivity and demonstrate the specific regulatory roles of Notch signaling pathways in the treatment of tumors.

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Disclosure of conflict of interest

None.

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